(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 June 2008 (26.06.2008)

PCT

(10) International Publication Number
WO 2008/075949 A1

(51) International Patent Classification:
A61P 1/00 (2006.01) *A61P 3/10* (2006.01)
A61P 3/04 (2006.01) *A61P 1/14* (2006.01)
A61P 9/00 (2006.01) *A61P 37/00* (2006.01)
A61P 9/10 (2006.01) *A61K 35/74* (2006.01)
A61P 3/08 (2006.01)

(21) International Application Number:
PCT/NL2007/050667

(22) International Filing Date:
18 December 2007 (18.12.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
06126673.0 20 December 2006 (20.12.2006) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

WO 2008/075949 A1

(54) Title: MODULATION OF HUMAN MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN (MTP OR MTTP) GENE EXPRESSION BY FOOD-GRADE/INGESTED DIETARY MICROORGANISMS

(57) Abstract: The present invention relates to the field of using microorganisms, especially food grade bacteria, to modulate intestinal MTP expression levels in order to treat and/or prevent weight gain, obesity, atherosclerosis, hyperglyceridaemia, hypercholesterolaemia, diabetes, dyslipidaemia and/or disorders associated with impaired intestinal immune response to antigens.

Modulation of human microsomal triglyceride transfer protein (MTP or MTTP) gene expression by food-grade/ingested dietary microorganisms

FIELD OF THE INVENTION

The present invention relates to the field of food-grade microorganisms, especially bacteria and/or their components. Especially, the use of food-grade microorganisms, which are capable of modulating intestinal MTP gene expression, for the preparation of food or feed compositions, food or feed supplements or pharmaceutical compositions for the treatment and/or prevention of a sub-optimal (or non-healthy) intestinal microbiota, weight gain, obesity, atherosclerosis, hyperglyceridaemia, hypercholesterolaemia, diabetes, dyslipidaemia and/or disorders associated with impaired intestinal immune response to antigens is provided herein. Also provided are compositions comprising one or more microbial strains or components thereof (such as cell fractions) which are capable of modulating MTP gene expression in human intestinal cells or tissues *in vivo* and *in vitro*, as are methods for isolating such strains and for establishing administration/dosage regimes for such strains. Furthermore, the use of intestinal MTP gene expression as a biomarker for the health status of humans and their associated intestinal microbiota is provided herein.

BACKGROUND OF THE INVENTION

Microsomal triglyceride transfer protein (MTP), which catalyzes the transport of triglyceride, cholesterol ester and phospholipids between phospholipids surfaces, is a heterodimer, consisting of an 88 kDa catalytic domain which is non-covalently associated with a 58 kDa PDI (protein disulfide isomerase). The human cDNA and genomic DNA encoding the large subunit MTP have been cloned and characterized (Sharp et al. 1993, Nature 365: 65-69). The human MTP gene was found to be primarily expressed in liver and intestinal tissue, which is compatible with its proposed function in triglyceride transfer (Hagan et al. 1994, J Biol Chem 269: 28737-28744).

MTP plays a role in the assembly and secretion of apolipoprotein B (apoB) containing lipoproteins and high plasma levels of these lipoproteins may be associated with atherosclerosis and coronary heart diseases. Humans carrying non-functional MTP genes have a condition referred to as abetalipoproteinaemia and a defective production

of apoB-containing lipoproteins. Hepatic over-expression of MTP results in an increased *in vivo* secretion of VLDL (very low density lipoprotein) triglycerides and apoB (Tietge et al. 1999, J. Lipid Res. 40: 2134-2139). MTP liver-specific knock-out mice results in an abrogation of VLDL/LDL production (Chang et al. 1999, J Biol Chem 274: 6051-6055). Similarly, it was shown that the inhibition of MTP activity causes a decrease in the secretion rate of apoB-containing lipoproteins in human and intestinal cells in vitro (Jamil et al. 1996, PNAS 93: 11991-11995; Van Greevenbroek et al. 1998, J Lipid Res 39: 173-185). These findings, therefore, suggest that MTP plays a role in modulating lipoprotein production in the liver and intestine.

Recently, MTP was found to be also involved in modulating the intestinal immune response to antigens. Lipid antigens are presented to T cells by CD1 molecules, which are major histocampatibility complex (MHC) class I-homologues. CD1 is expressed on myeloid cells, hepatocytes and intestinal epithelial cells. The type I CD1 molecules (CD1a, CD1b and CD1c) are expressed on dendritic cells in the intestinal mucosa. It was shown previously that MTP regulates CD1d function and, hence, natural killer T (NKT) cell biogenesis (Brozovic et al. 2004, Nature Med 10: 5: 535-9). Recent observations that MTP is involved in antigen presentation, and is able to regulate CD1a, CD1b and CD1c production, suggest that MTP is important in the host response to microbial pathogens. The presence of type 1 CD1 molecules on dendritic cells indicates a putative role of MTP in the pathogenesis of mucosal inflammation-related disorders (Kaser A, Hava D, Yoshida M, Kuo T, Nagaishi T, Dougan S, Lugt Vander B, Haddad W, Brenner M, Blumberg R. Microsomal triglyceride transer protein regulates endogenous and exogenous antigen presentation by group 1 Cd1 molecules. Gastroenterology 2006;130: 4 suppl 2; 126). Furthermore, recent data suggest that MTP regulates an intestinal barrier-protective CD1d pathway that is mediated by NKT cells (Kaser A, Yoshida M, Furuta G, Zhu F, Davidson N, Colgan S, Blumberg R. Intestinal Microsomal Triglyceride Transfer Protein (MTP) regulates Cd1d function on the intestinal epithelium and protects from mortality in the oxazolone model. Gastroenterology 2006;130: 4 suppl 2; 126).

The regulation of MTP gene expression seems to be complex. In hamsters, high fat and high cholesterol diets have been shown to up-regulate hepatic MTP mRNA levels. *In*

vitro studies on human hepatoma cells have shown that ethanol (Lin et al. 1997, FASEB J. 11, 1145-1152), fresh garlic (Lin et al. 2002, J Am Soc Nutr Sci, 132: 1165-1168) and insulin (Lin et al. 1995, J Lipid Res 36: 1073-1081) down-regulate hepatic MTP expression.

Some drugs, such as diaminoindanes and benimidazole-based compounds, with MTP-inhibitory activity are under investigation for the treatment of hyperlipidemia (Burnett JR 2006, IDrugs, Jul;9(7):495 -9; Chandler CE, Wilder DE, Pettini JL, Savoy YE, Petras SF, Chang G, Vincent J, Harwood HJ Jr, J Lipid Res. 2003 44(10):1887-901). These drugs mostly inhibit both liver and the intestinal MTP expression and may result in fatty livers. Intestinal-specific MTP inhibitors would be desirable, preferably ones which are based on natural food-grade products. Fresh garlic seems to contain MTP inhibiting substances which are relatively specific for inhibiting intestinal MTP expression in rats, 3 hours after oral administration (Lin MC, Wang EJ, Lee C, Chin KT, Liu D, Chiu JF, Kung HF, J Nutr. 2002 Jun;132(6):1165-8).
However, the components, which are responsible for this effect, have not yet been identified and it is not clear whether the effect and specificity would be the same in humans. Furthermore, the addition of large quantities of fresh garlic to food products would limit the applicability to certain types of food.

It is, therefore, an object of the invention to provide alternative, food grade MTP-expression modulators, especially MTP gene expression inhibitors and activators, methods for identifying and isolating these and compositions comprising these. It is a further object of the invention to provide a method for evaluating and monitoring the health status of the human intestinal microbiota, and means for distinguishing between a healthy intestinal microbiota and an unhealthy or sub-optimally healthy intestinal condition. In addition, means for treating and/or preventing a sub-optimal or abnormal intestinal microbiota are provided, whereby a sufficient amount of a MTP-gene expression modulating composition, comprising or consisting of at least one microorganism capable of modulating intestinal MTP-gene expression, is administered.

GENERAL DEFINITIONS

"Lactic acid bacteria" and "lactic acid producing bacteria", is used herein interchangeably and refers to bacteria, which produce lactic acid as an end product of fermentation, such as, but not limited to, bacteria of the genus *Lactobacillus*, *Streptococcus, Lactococcus, Oenococcus, Leuconostoc, Pediococcus, Carnobacterium, Enterococcus*. In addition, *Bifidobacterium* and *Propionibacterium* species are considered for this application to belong to lactic acid bacteria although they have a distinct phylogenetic position.

"Probiotics" or "probiotic strain(s)" refers to strains of live or viable micro-organisms, preferably bacteria, which when administered in adequate amounts provide a health benefit to the host subject, e.g. when ingested (e.g. orally, enterally or by inhalation) by a subject. Probiotics are defined as "viable microbial food supplements which, when taken in the right doses beneficially influence human health" (Salminen et al. 1998, WHO 2002).

"Micro-organisms" include bacteria and fungi, such as yeasts. When reference herein is made to bacteria, it is understood that the embodiments also apply to other microorganisms.

"Enteral" refers herein to the delivery directly into the gastrointestinal tract of a subject (e.g. orally or via a tube, catheter, capsules or stoma).

"Food-grade" micro-organisms are in particular organisms, which are considered as not harmful, when ingested by a human or animal subject.

"Components" of microorganisms or "inactivated" microorganisms refers to non-viable microorganisms, such as dead cells, cell fragments, and the like.

A "subject" refers herein to a human or animal, in particular a vertebrate, such as but not limited to domestic animals.

The term "comprising" is to be interpreted as specifying the presence of the stated parts, steps or components, but does not exclude the presence of one or more additional parts, steps or components. A composition comprising a lactic acid bacterium may thus comprise additional bacterial strains etc. However, a lactic acid bacterium or a mixture of several distinct lactic acid bacteria is preferably a main active component of a composition of the invention. More preferably, a lactic acid bacterium or a mixture of several distinct lactic acid bacteria is the sole active component of a composition of the invention.

"Percentage" or "average" generally refers to percentages of averages by weight, unless otherwise specified or unless it is clear that another basis is meant. For example, when referring to % gene expression, it is clear that the relative or absolute amount of a gene transcript being produced is referred to.

The term "a" or "an" does not limit to one, but is interpreted as at least one. Therefore, when reference is made to 'a bacterium' it is understood that a plurality of bacteria are encompassed.

The term "derivative" refers to the biological material that represents a substantially unmodified copy of the material, such as material produced by growth of micro-organisms, e.g. growth of bacteria in culture media. The term "derivative" also includes material created from the original micro-organism which retains the beneficial properties of the unmodified strain, but which is modified to have new additional properties, for example caused by heritable changes in the genetic material. These changes can either occur spontaneously or be the result of applied chemical and/or physical agents (e.g. mutagenesis agents) and/or by recombinant DNA techniques as known in the art.

The term "gene" means a DNA comprising a region (transcribed region), which is transcribed into an RNA molecule (e.g. an mRNA) in a cell, operably linked to suitable regulatory regions (e.g. a promoter). A gene may thus comprise several operably linked fragments, such as a promoter, a 5' leader sequence, a coding region and a 3'nontranslated sequence comprising a polyadenylation site. An "endogenous gene" is a gene found in its natural environment in the cells of an organism.

"Expression of a gene" refers to the process wherein a DNA region which is operably linked to appropriate regulatory regions, particularly a promoter, is transcribed into an RNA, which is biologically active, i.e. which is capable of being translated into a biologically active protein or peptide.

A "transcription regulatory sequence" is herein defined as a nucleic acid sequence that is capable of regulating the rate of transcription of a (coding) sequence operably linked to the transcription regulatory sequence. A transcription regulatory sequence as herein defined will thus comprise all of the sequence elements necessary for initiation of transcription (promoter elements), for maintaining and for regulating transcription, including e.g. attenuators or enhancers. Although mostly the upstream (5') transcription

regulatory sequences of a coding sequence are referred to, regulatory sequences found downstream (3') of a coding sequence are also encompassed by this definition.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter, or rather a transcription regulatory sequence, is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein encoding regions, contiguous and in reading frame.

"Modulation" of gene expression refers to an up-regulation or down-regulation of gene expression relative to one or more control subjects or control samples. It is understood that the up-regulation or down-regulation should be statistically significant, taking account of normal variation in expression levels.

The term "induced" or "up-regulated" gene expression as used herein refers to the induction of activity of a transcription regulatory sequence (especially a promoter) and the resulting increase in mRNA transcription of the nucleic acid sequence operably linked thereto. Thus, in particular, an initiation or increase in mRNA transcript production of a DNA sequence operably linked to the transcription regulatory sequence is referred to. Induction or up-regulation refers therefore to a change from no mRNA transcription to mRNA transcription, or to a change from a certain transcription level to a higher transcription level. In particular, an increase of at least 5%, 10%, 20%, 30%, 50%, or more, of mRNA transcription after induction is encompassed in this definition.

Vice versa, "inhibition" or "down-regulation" or "reduction" of gene expression as used herein refers to the reduction of activity of a transcription regulatory sequence (especially a promoter) and the resulting reduction or inhibition in mRNA transcription of the nucleic acid sequence operably linked thereto. Thus, in particular, a down-regulation or inhibition of mRNA transcript-production of a DNA sequence operably linked to the transcription regulatory sequence is referred to. Down-regulation or inhibition refers therefore to a change from a certain level of mRNA transcription to a lower or no transcription level. In particular, an reduction of at least 5%, 10%, 20%, 30%, 50%, or more, of mRNA transcription after inhibition is encompassed in this definition.

"Tissue-specific down-regulation" refers to the down-regulation of transcription in a specific tissue, such as the intestinal cells.

"MTP inhibitor" refers to one or more microorganisms or compositions comprising these (or components of these) which are capable of down-regulating or inhibiting MTP expression after contact of a sufficient amount of the microorganim(s) with intestinal cells or tissue (either directly or indirectly, by e.g. signal transduction pathways), for a sufficient period of time. "MTP activator" or "MTP inducer" refers to one or more microorganisms or compositions comprising these (or components of these) which are capable of up-regulating MTP expression after contact of a sufficient amount with intestinal cells or tissue for a sufficient period of time.

A "sufficient period" refers to an exposure time (i.e. contact with intestinal cells) which is sufficient to result in the upregulation or downregulation of MTP gene expression. Routine experimentation can be used to determine a sufficient amount and a sufficient exposure period to achieve the desired modulation of MTP expression.

"Tissue-specific MTP inhibitor" refers to microorganisms or compositions which are capable of down-regulating or inhibiting MTP expression in a specific tissue or cell type. *Vice versa*, "tissue specific MTP activator" or "MTP inducer" refers to microorganisms or compositions which are capable of inducing MTP expression in a specific tissue or cell type.

"Stringent hybridization conditions" can also be used to identify nucleotide sequences, which are substantially identical to a given nucleotide sequence (such as a probe). Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequences at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically stringent conditions will be chosen in which the salt concentration is about 0.02 molar at pH 7 and the temperature is at least 60°C. Lowering the salt concentration and/or increasing the temperature increases stringency. Stringent conditions for RNA-DNA hybridizations (Northern blots using a probe of e.g. 100nt) are for example those which include at least one wash in 0.2X SSC at 63°C for 20min, or equivalent conditions. Stringent conditions for DNA-DNA hybridization (Southern blots using a probe of e.g. 100nt) are for example those which include at least one wash (usually 2) in 0.2X SSC

at a temperature of at least 50°C, usually about 55°C, for 20 min, or equivalent conditions.

The term "substantially identical", “substantial identity” or “essentially similar” or “essential similarity” or “variant” means that two peptide or two nucleotide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default parameters, share at least about 80 percent sequence identity, preferably at least about 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). GAP uses the Needleman and Wunsch global alignment algorithm to align two sequences over their entire length, maximizing the number of matches and minimizes the number of gaps. Generally, the GAP default parameters are used, with a gap creation penalty = 50 (nucleotides) / 8 (amino acids sequence) and gap extension penalty = 3 (nucleotides) / 2 (amino acid sequence). For nucleotides the default scoring matrix used is nwsgapdna and for proteins the default scoring matrix is Blosum62 (Henikoff & Henikoff, 1992). When two sequences are of similar length, they are preferably optimally aligned using a global alignment algorithm (Needleman and Wunsch), while when sequences are of different lengths a local alignment algorithm is preferred (Smith and Waterman; using a Gap creation penalty of 10.0 and a gap extension penalty of 0.5, and the default scoring matrix as above), in order to determine sequence identity. It is clear than when RNA sequences are said to be essentially similar or have a certain degree of sequence identity with DNA sequences, thymine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence.

DETAILED DESCRIPTION

The present inventors found that administration of certain probiotic bacterial species (e.g. *L. plantarum* strain WCFS1) directly into the small intestinal lumen of human healthy subjects *in vivo*, resulted in significant modulation of gene expression of a large number of genes in small intestinal mucosa. Microarray analysis of a short term exposure study and a long term exposure study showed that the expression of one gene in particular, encoding human microsomal triglyceride transfer protein (MTP), was down-regulated in mucosal biopsies taken after 1 hour exposure to the bacterial strain (short term exposure), while it wasup-regulated in mucosal biopsies taken after 6 hours exposure to the bacterial strain (long term exposure). This was confirmed by the

proteome analyses on the samples of the 6-hours exposure study. These analyses showed that two proteins differed consistently in all volunteers. One of these protein spots was identified as human microsomal triglyceride transfer protein (MTP), while this analysis failed to allow identification of the second spot. These two studies showed that MTP mRNA levels were not only modulated (a decrease followed by an increase) by contact of intestinal cells with the bacteria, but that MTP protein levels were modulated (a decrease followed by an increase) as well.

In a third intervention study, the investigators showed that intermittent oral intake of the bacterial strain for 6 h increased the expression of the MTP gene in the same order of magnitude as observed in the 6-h intraluminal exposure experiment. This study thus confirmed that ingestion of live or viable food-grade bacteria and exposure of the intestinal cells or tissues to these bacteria for a sufficient period of time leads to a significant modulation of MTP expression and MTP protein levels in the intestine.

It came as a surprise that administered intestinal bacteria were capable of significantly and consistently modulating MTP expression levels in intestinal tissue, as MTP expression was thought to be regulated by dietary compounds such as fat and cholesterol.

In the third intervention study, also non-viable (killed by heating), and mid-log harvested bacteria were administered orally at 30-min intervals to healthy volunteers during a 6-h period. While intake of the stationary phase cells of the probiotic strain resulted in a significantly increased expression of the MTP gene by 13.9 % ($P = 0.179$), the inventors did not observe an effect of the orally ingested non-viable cells. For the mid-log harvested live bacteria an increase in MTP expression of 6.8 % was observed, but the investigators do not consider this minor change to have a biological significance.

It is, therefore, a preferred embodiment to use food grade, live and/or viable microorganisms in the methods and products of the invention. It is another preferred embodiment to use stationary phase cultures of the microorganisms. However, neither is essential, as for other microorganisms (e.g. other food-grade bacterial genera, species or strains) it may be feasible to use dead / non-viable cells and/or cell components as modulators of MTP expression and/or to use a different growth phases of the microorganism(s) (e.g. mid-log phase or late log-phase). The testing of which

microorganisms or combinations thereof (which genera, species or strains, which growth phase and/or live or viable versus dead or non-viable) provide the desired effect (up-regulation or down-regulation) on MTP expression involves routine experimentation and can be carried out using undue efforts, as described herein.

The finding that live or viable microorganisms are capable of modulating intestinal MTP expression allowed the inventors to prepare food, feed or pharmaceutical compositions, comprising or consisting of at least one microorganism which is capable of modulating MTP-gene expression. Such compositions are suitable for intestinal MTP gene activation or inhibition, depending on what kind of modulation of MTP gene expression is required. Methods for identifying the microorganism(s) which have the desired effect on MTP gene expression and for using these to make compositions having the desired effect are described further herein below.

Such MTP-activator or MTP-inhibitor compositions are suitable for treating and/or preventing a variety of diseases and disorders associated with (too) high or (too) low MTP gene expression, such as a sub-optimal intestinal microbiota (e.g. in patients having intestinal MTP-expression levels which are higher or lower than in healthy subjects), or weight gain, obesity, atherosclerosis, hyperglyceridaemia, hypercholesterolaemia, diabetes and/or dyslipidaemia (which are associated with abnormally high MTP gene expression and would benefit from MTP-inhibitor compositions), or abetalipoproteinemia, which is associated with mutations in the MTP gene (Di Leo E, et al. Atherosclerosis 2005 180(2):311-8) and hence, with low levels of functional MTP gene expression (and would benefit from MTP-activator compositions). In addition, subjects suffering from an impaired intestinal immune response and/or an increased progression or severity of an impaired intestinal immune response, and/or subjects susceptible to intestinal epithelial damage would benefit from administration of an MTP activator composition.

In addition, new methods for identifying microbial modulators of MTP expression, especially MTP activators or MTP inhibitors, are provided.

The different embodiments of the invention are linked, and features described in one embodiment are understood to also apply to the other embodiments, unless stated otherwise.

Uses according to the invention

In one embodiment of the invention provides the use of at least one food grade microorganism, preferably a bacterium (or component thereof), capable of modulating Microsomal Triglyceride Transfer Protein (MTP) gene expression in human intestinal tissue or intestinal cells for the preparation of a composition for the treatment or prevention of weight gain, obesity, atherosclerosis, hyperglyceridaemia, hypercholesterolaemia, diabetes, dyslipidaemia and/or disorders associated with impaired intestinal immune response to antigens.

In a further embodiment, the use of at least one food grade microorganism, preferably a bacterium (or component(s) thereof), capable of modulating Microsomal Triglyceride Transfer Protein (MTP) gene expression in human intestinal tissue or intestinal cells for the preparation of a composition for the treatment or prevention of an abnormal or sub-optimal intestinal microbiota compared to healthy subjects.

The microorganism (e.g. bacterium), or a suitable amount thereof (or a suitable amount and type of component thereof), is capable of either upregulating or downregulating MTP gene expression in human intestinal tissue or cells *in vivo* and/or *in vitro*, following exposure of the intestinal tissue/cells to the microorganism(s), or compositions comprising these, in a sufficient amount and for a sufficient period of time A short term exposure is preferably used to obtain a downregulation of MTP as defined herein. Short term exposure preferably means approximately one hour exposure. One hour exposure is preferably carried out as described in the examples (1.2. description of study 1). A long term exposure is preferably used to obtain an upregulation of MTP as defined herein. Long term exposure preferably means approximately six hour exposure. Six hour exposure is preferably carried out as described in the examples (1.3. description of study 2 or 1.4. description of study 3).Exposure of the intestinal tissue/cells to the microorganim(s) is preferably carried out by using an enteric composition or a composition which is suitable for enteric administration (via catheter or a tube). In an alternative preferred embodiment, the

exposure of the intestinal tissue/cells to the microorganism(s) is carried out by using a composition suitable for oral administration. Such composition is preferably an enteric composition. Enteric compositions are already known in the prior art. Such microorganisms already exist in the art (e.g. *L. plantarum* WCFS1) or can be identified and isolated using methods described herein. For example, the probiotic bacterium *Lactobacillus plantarum* strain WCFS1 described in the Examples can significantly modulate (especially up-regulate) MTP expression in mucosal cells of the small intestine and is, therefore, suitable for preparing compositions which are capable of modulating MTP expression of intestinal tissue/cells. A significant modulation of MTP gene expression refers herein to either an increase or decrease in absolute or relative MTP gene expression by at least about 5%, preferably 10%, 15%, 20%, 30%, 40% or more, relative to a suitable control (such as MTP expression in a biological intestinal sample taken prior to contact with the bacterium).

One can test whether or not a microorganism (e.g. a bacterium) has the capability to significantly upregulate or downregulate intestinal MTP gene expression, for example by determining the relative or absolute amount of MTP transcript prior to or at contact with a microorganism or components of a microorganism (e.g. in the form of a suspension) and at one or more time points after contact. Alternatively, the MTP transcript levels may be compared after contact with a microorganism (or component) with the MTP transcript levels after contact with a suitable control composition (e.g. lacking the microorganism or component). One easy method for determining the capability of a microorganism to modulate MTP gene expression is to provide at least two biological samples of intestinal tissue or cells (e.g. mucosal cells of the small intestine) e,g, one taken before and one after contact with the microorganism and/or at different time points after contact with the microorganism and/or one taken after contact with the microorganism and one after contact with a suitable control composition (e.g. lacking the microorganism) and to assay and compare the MTP transcript levels in such samples. MTP mRNA levels in different samples and/or at different time points can for example be compared and quantified using quantitative PCR. See also further below.

In the same way, one can determine the amount of the microorganism(s) which is sufficient for upregulating or downregulating MTP expression levels and/or exposure period which is sufficient for inducing the desired modulation of MTP expression in a consistent way. For example, a continuous exposure (e.g. for more than 1, 2, 3, 4, 5, 6, or more hours), or a single exposure of one dosage unit, or several intermittent exposures (e.g. once every 10, 15, 20, 25, 30 minutes, hour, or day, etc.) of the intestinal tissue/cells may suffice to induce or inhibit MTP expression. One can simply test exposure to different amounts (dosages) of microorganisms and/or to different exposure periods to determine the optimal amounts and/or periods of contact (i.e. dosage units over time) for achieving the desired effect on MTP gene expression.

The food grade microorganisms which is used for making the composition is preferably a food grade probiotic microorganisms (e.g. especially bacterium) and/or a food grade lactic acid bacterium. Any food grade microorganism, which is capable of modulating MTP expression may be used. Preferred food grade fungi/yeasts are of the genus *Saccharomyces*, *Pichia*, or *Hansenula, Rhizopus, Aspergillus*. Preferred food grade bacteria belongs to a genus selected from the group consisting of *Lactobacillus*, *Lactococcus, Leuconostoc, Carnobacterium, Streptococcus, Bifidobacterium, Bacteroides, Eubacterium, Clostridium, Fusobacterium, Propionibacterium, Enterococcus, Staphylococcus, Peptostreptococcus,* and *Escherichia*. See also further below. Thus, any food grade species or strain of bacteria, yeast or fungi may be tested for its capability of modulating MTP expression of human intestinal cells or tissue. Also mixtures of microorganisms may be tested for their capability of modulating MTP gene expression, and used for making compositions. Two or more microorganisms may for example have a synergistic effect when used together (i.e. the effect on MTP-gene expression of the combination of microorganisms is larger than the sum of the effects of each microorganisms administered on its own).

Once one or more suitable microorganisms, or mixtures of microorganisms, (or components thereof) have been identified, standard methods can be used for making a composition for the treatment or prevention of one of the above diseases or disorders or for improving the microbiota of the intestine and/or preventing or treating an abnormal or sub-optimal microbiota (having an intestinal MTP-gene expression which deviates

significantly from that of normal, healthy intestines). Such methods are described further below.

In principle, the microorganism is grown on a suitable medium under suitable growth conditions. The microorganism may then be used together with the growth medium or removed from the medium (e.g. by centrifugation or filtration). In a preferred embodiment, the microorganism(s) used is grown to stationary phase, although microorganisms grown to other phases may also suitably be used, for example log and/or mid-log phase (exponential phase) and/or late log phase and/or stationary phase microorganisms. When mixtures are used, microorganisms may be grown separately and mixed subsequently in defined amounts, or they may be grown together as a co-culture. Most preferably, live or viable (e.g. lyophilized) microorganisms are used for making the compositions according to the invention.

In one embodiment also components of the microorganism or its culture (e.g. bacterial components, such as cell fragments, or bacterial culture components) may be used, whereby the components and/or culture have the capability of modulating MTP expression as described.

Also mixtures may be used, for example mixtures of one or more microorganism (see above; e.g. one or more bacterial strains), or of one or more bacterial components or different mixtures of bacterial components, or mixtures comprising or consisting of one or more bacterial strains and/or one or more bacterial components and/or bacterial culture components. Two or more bacteria (or components) capable of inducing MTP expression may be used together. Similarly, two or more bacteria (or components) capable of down-regulating MTP expression may be used together in the preparation of one composition or in the preparation of two or more discrete dosage units for separate (e.g. simultaneous or consecutive) intake or administration.

In all embodiments defined in this section, a food-grade bacterium is preferably the main active component of a composition of the invention. It means that the effect induced by a composition of the invention (i.e. modulation of MTP gene expression) is mainly due to the presence of a food-grade bacterium. More preferably, when a composition of the invention is to be used for treating or preventing atheroslerosis,

tannin is not present in a composition of the invention. Even more preferably, tannin is not present in a composition of the invention.

Methods according to the invention

In another embodiment of the invention a method for identifying and/or selecting a microorganism (or a mixture of microorganisms), preferably a food-grade bacterium, capable of modulating the expression levels of the human MTP gene is provided. The method comprises the steps of:

(a) contacting a composition comprising or consisting of one or more microorganisms or components thereof, preferably a food-grade bacterium, with intestinal tissue or intestinal cells of a human subject *in vitro*; or using one or more intestinal tissue or cell samples (e.g. biopsies, a biological sample) of a subject before and/or after contact with said composition *in vivo*;

(b) determining the expression level of the gene encoding human Microsomal Triglyceride Transfer Protein (MTP) in said intestinal tissue or cells at one or more time points before and/or after contact; and

(c) identifying and/or selecting a microorganism (or a mixture of microorganism, or components thereof) which upregulates or downregulates MTP expression in said intestinal tissue or cells compared to the expression levels in a suitable control (e.g. the intestinal tissue or cells prior to contact or at an earlier timepoint after contact or contacted with a suitable control composition lacking the microorganism(s) or components).

The method may be carried out *in vivo* or *in vitro*. The *in vivo* method is carried out by administering a composition comprising or consisting of one or more specific microorganisms (or components thereof) to a subject, e.g. orally or via a catheter directly to a part of the intestine (such as the small intestine) for a certain period of time (e.g. continuous or at one or more time intervals) and by obtaining or providing a tissue sample from said subject, especially from the tissue type contacted with the microorganisms.

The subjects are preferably healthy human subjects, or at least subjects which have no gastrointestinal illness. Preferably, several subjects are administered with the test

composition and, on another test day, with a suitable control composition in a randomised fashion. The subjects may be of any sex and age.

In the *in vitro* method either a tissue sample is provided, which was contacted with the microorganisms for a suitable period of time, or human cell lines are cultured and used in the assay. Suitable tissue samples are those cell types contacted with the micro-organism, such as cells of the small intestine. Suitable cell lines are intestinal cell lines, such as Caco-2 cells (available e.g. at the American Type Culture Collection), HT29, HT29-MTX, or Int407, which can be cultured using known methods and contacted with the composition comprising the microorganism. "Contacted" means the physical contact between the tissue or cells and the microorganisms for a suitable period of time. Contact can be brought about by ingestion and passage into the intestine or by direct application to certain parts of the intestine or direct application to cultured tissue or cultured cells. The term 'contact' or 'contacting' may also encompass tissues and/or cells which are not in direct physical contact with the microorganism(s) or compositions, but which were indirectly contacted, for example through signal transduction pathways (for example, cells or tissues in the vicinity of directly-contacted cells are encompassed herein).

The microorganism used in (a) may be any microorganism or mixture of microorganisms. In one embodiment preferably a single strain per composition is tested, although also mixtures of several strains (2, 3, 4, 5, 10, 15, 20 or more) may be used. Most preferably, food grade strains are used, especially food grade probiotic or lactic acid bacteria. The strains used in step (a) may be one or more known strains, such as strains obtainable from deposit institutions or they may not exist in isolated form yet. For example, they may be present in yoghurt, milk or other compositions. Therefore, the starting composition used in step (a) may be a food composition, such as a dairy composition, already comprising a plurality of strains or to which one or more strains are added. It is understood that "food" includes herein not only solid compositions, but also semi-solid and liquid compositions, such as beverages. A suitable comparative strain may be the probiotic *L. plantarum* strain WCFS1 used in the examples (Accession number NCIMB8826, National Collection of Industrial and Marine Bacteria, Aberdeen, U.K).

Preferably, the composition comprising said one or more microorganisms are in liquid or semi-liquid form. The strain(s) may be grown to a suitable density on growth medium as known in the art, optionally extracted from the medium (e.g. by centrifugation) and then suspended in a liquid solution (e.g. a saline solution). The amount of micro-organism used may vary depending on the genus and species or on the strain, but generally for a bacterial or yeast strain at least about 10^6 or 10^7, e.g. about 10^7-$10^{12,}$ 10^7-10^{13} or 10^7-10^{15}, or more, cells may be suitable. The microorganism's cells may be suspended in a suitable volume of liquid and may then be contacted with the intestinal cells/tissue, and the effect on MTP gene expression may be assessed after a suitable period of time.

Most preferably the microorganism is a food grade bacterium, especially a lactic acid bacterium or any probiotic bacterium. Other preferred microorganisms are food-grade fungi and yeasts, such as bakers or brewers yeast, such as species of the genus *Saccharomyces*, *Pichia*, or *Hansenula, Rhizopus, Aspergillus*. A preferred bacterium belongs to a genus selected from the group consisting of *Lactobacillus*, *Lactococcus, Leuconostoc, Carnobacterium, Streptococcus, Bifidobacterium, Bacteroides, Eubacterium, Clostridium, Fusobacterium, Propionibacterium, Enterococcus, Staphylococcus, Peptostreptococcus* and *Escherichia*.

A further preferred bacterium is a *Lactobacillus* or *Bifidobacterium* species selected from the group consisting of *L. reuteri, L. fermentum, L. acidophilus, L. crispatus, L. gasseri, L. johnsonii, L. plantarum, L. paracasei, L. casei, L. sakei, L. murinus, L. jensenii, L. salivarius, L. minutis, L. brevis, L. gallinarum, L. amylovorus, B. bifidum, B. longum, B. infantis, B. breve, B. adolescentis, B. animalis, B. gallinarum, B. magnum, B. thermophilum and B. lactis*.

In step (b) of the method, the expression level of the MTP gene is determined in the tissue or cells at one or more time-points following contact. This can be done by a range of (preferably quantitative or semi-quantitative) molecular techniques available in the art, such as RT-PCR (e.g. quantitative RT-PCR) using (degenerate or specific) primer pairs which amplify part of the human MTP transcript (mRNA or the corresponding cDNA), nucleic acid hybridization methods (e.g. using nucleic acid

probes / oligonucleotides which are complementary to part of the MTP mRNA or cDNA). Optionally in addition or as an alternative, the MTP protein level may be analyzed using e.g. SDS-page and Western blots, ELISA assays, immunocytological assays. The Examples show that also MTP protein levels were modulated, in correlation with MTP transcript levels. Therefore, a significant up- or down-regulation of MTP expression can also be determined by assessing whether MTP protein levels are significantly changed following contact with the microorganism (or components thereof).

The human MTP cDNA is shown in SEQ ID NO: 1 (EMBL Accession number X91148) and the protein in SEQ ID NO: 2 (CAA62585.1). In step (b) the relative amount of SEQ ID NO: 1, or of a nucleic acid sequence comprising at least 80, 85, 90, 95, 98%, 99% or more nucleic acid sequence identity to SEQ ID NO: 1 (referred herein to as "variants" of SEQ ID NO: 1; whereby the % identity is determined over the entire length, using the Needleman and Wunsch algorithm with the parameters defined above), is determined, relative to the amount at either an earlier time-point or in a suitable control treatment (e.g. tissue or cells contacted with the same composition for the same period of time under the same conditions, but lacking the micro-organism or components thereof). As mentioned, the presence and amount of SEQ ID NO: 1 or variants thereof can be determined using various known techniques. For example PCR primer (specific or degenerate) can be designed which amplify all or part of SEQ ID NO: 1 (or variants thereof). Similarly, probes which hybridize with SEQ ID NO: 1 (or variants), e.g. under stringent conditions, can be made and used. For example, the Human Genome Gene Chip U133A of Affymetrix contains probes of SEQ ID NO: 4-14, which detect the target MTP transcript sequence fragment of SEQ ID NO: 3 (and detects thereby SEQ ID NO: 1 or a variant thereof), whereby no noteworthy cross-hybridization for this probe set is found.

In a preferred method, the modulation of an intestinal MTP gene expression is determined in step b) by analyzing the level of SEQ ID NO: 1, or of a nucleic acid sequence comprising at least 80% sequence identity to SEQ ID NO: 1, in said tissue or cells.

In step (c) one then identifies and/or selects the microorganism (or the composition comprising the microorganism, and/or its components) which significantly upregulates or downregulates the MTP gene expression level compared to the expression level in a suitable control. As mentioned above, a "significant reduction" of intestinal MTP gene expression is a reduction in mRNA levels by at least about 5%, preferably 10%, 15%, 20%, 30%, 40% or more, relative to the control. Similarly, a "significant upregulation" of intestinal MTP gene expression is a increase in mRNA levels by at least about 5%, preferably 10%, 15%, 20%, 30%, 40% or more, relative to the control. Standard statistical method can be used in analyzing the expression data.

If mixtures of microorganisms are used to start with, one may need to repeat steps (a)-(c) several times using fractions of the original composition or one may need to separate the mixture of microorganisms first and then repeat steps (a)-(c) in order to identify which microorganism of the mixture was responsible for the modulated MTP expression.

Microorganisms according to the invention

The isolated microorganism(s) identified using the above method, and compositions comprising one or more thereof, are also embodiments of the invention. The microorganism(s) is/are capable of modulating, especially substantially up- or down-regulating MTP expression in human intestinal cells when contacted with the cells in a suitable amount and for a suitable period of time. Preferably, the effect on MTP expression is specific for the intestinal MTP gene, while the hepatic MTP gene expression is not affected (following contact between the microorganism and the intestine of a subject).

The microorganisms, preferably food grade bacteria, which have this *in vivo* (and/or *in vitro*) functionality, are preferably used in the preparation of a composition for the treatment and/or prevention of one or more diseases or disorders selected from: weight gain, obesity, atherosclerosis, hyperglyceridaemia, hypercholesterolaemia, diabetes, dyslipidaemia and/or disorders associated with impaired intestinal immune response to antigens, as described herein above. In addition, the microorganism(s) are used in the preparation of a composition for the treatment and/or prevention of an abnormal or sub-

optimal intestinal microbiota and for bringing intestinal MTP gene expression back to a normal level.

Compositions according to the invention

Provided are food or feed composition or food or feed supplements, as well as pharmaceutical compositions, comprising a suitable amount of at least one microorganism or its component(s) selected from:

(a) a microorganisms (or its component) capable of significantly down-regulating intestinal MTP expression in a human subject *in vivo* (and/or *in vitro*) and

(b) a microorganisms (or its component) capable of significantly up-regulating intestinal MTP expression in a human subject *in vivo* (and/or *in vitro*).

In all embodiments defined in this section, a microorganism, i.e. a food-grade bacterium is preferably the main active component of a composition of the invention. It means that the effect induced by a composition of the invention (i.e. modulation of MTP gene expression) is mainly due to the presence of a microorganism, i.e. a food-grade bacterium. More preferably, when a composition of the invention is to be used for treating or preventing atheroslerosis, tannin is not present in a composition of the invention. Even more preferably, tannin is not present in a composition of the invention.

A further aspect of the invention relates methods for the production of such compositions. Strains according to the invention, preferably bacteria, are cultured under appropriate conditions, optionally recovered from the culture medium and optionally formulated into a composition suitable for the intended use.

Methods for the preparation of such compositions are known *per se*. Compositions for enteral or oral administration may be either food or food-supplement compositions or pharmaceutical compositions. Pharmaceutical compositions will usually comprise a pharmaceutical carrier in addition to the microorganisms of the invention. The preferred form depends on the intended mode of administration and (therapeutic or prophylactic) application. The pharmaceutical carrier can be any compatible, nontoxic substance suitable to deliver the microorganisms (or its components) to the GI-tract of a subject. E.g. sterile water, or inert solids may be used as the carrier usually

complemented with pharmaceutically acceptable adjuvants, buffering agents, dispersing agents, and the like. Compositions will either be in liquid, e.g. a stabilized suspension of the cells, or in solid forms, e.g. a powder of lyophilized host cells. As an example, for oral administration, the microorganisms, especially bacterial cells, can be administered in solid dosage forms, such as capsules, tablets, encapsulates, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. The microorganisms can be encapsulated in gelatin capsules together with inactive ingredients and powdered carriers, such as e.g. glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like.

A preferred composition according to the invention is suitable for consumption by a subject, preferably a human or an animal. Such compositions may be in the form of a food supplement or a complete food or food composition, which besides the microorganism(s) of the invention (or its components) also contains a suitable food base. A food or food composition is herein understood to include liquids for human or animal consumption, i.e. a drink or beverage. The food supplement, food or food composition may be a solid, semi-solid and/or liquid food or food composition, and in particular may be a dairy product, such as a milk, a fermented dairy product, including but not limited to a yoghurt, a yoghurt-based drink, cheese or buttermilk. Dairy product such as (plain and/or flavoured) milk may be in the form of powdered formulations (including milk powder, infant formula, etc.) and/or concentrated formulations (Sweet Condensed Milk, i.e. SCM, or Evaporated milk). Cheese may be fresh cheese, hard cheese, semi-hard cheese, cream cheese or a cheese product (processed cheese).Examples of liquid food supplement, food or food composition may further be a beverage such as a fruit-based beverage or a soya-based beverage. Examples of semi-solid food supplement, food or food composition include dressing, spread or topping. Examples of solid food supplement, food or food composition include coating, ice cream, cereal, cereal bars. Such foods or food compositions may be prepared in a manner known *per se*, e.g. by adding microorganism(s) of the invention to a suitable food or food base, in a suitable amount. In a further preferred embodiment, the microorganisms are used in or for the preparation of a food or food composition, e.g. by fermentation. Examples of such microorganisms include baker's or brewer's yeast

and lactic acid bacteria, such as probiotic lactic acid strains. In doing so, the microorganisms of the invention may be used in a manner known *per se* for the preparation of such fermented foods or food compositions, e.g. in a manner known *per se* for the preparation of fermented dairy products using lactic acid bacteria. In such methods, the microorganisms of the invention (or its components) may be used in addition to the micro-organism usually used, and/or may replace one or more or part of the micro-organism usually used. For example, in the preparation of fermented dairy products such as yoghurt or yoghurt-based drinks, a food grade lactic acid bacterium of the invention may be added to or used as part of a starter culture or may be suitably added during such a fermentation.

Preferably, the above compositions will contain the microorganism (or one or more of its components) in amounts that allow for convenient (oral) administration of the cells/components, e.g. as or in one or more doses per day or per week. In particular, the preparations may contain a unit dose of the microorganisms or its components.

Thus, the compositions can be in a form for separate administration, such as a capsule, a tablet, a powder, a gel, or a similar form. The dosage form comprises preferably a unit dose of the strain. Suitable dosages are 1×10^6 to 1×10^{12}, preferably 1×10^8 to 5×10^{10} colony forming units per dose, or the equivalent dosage of non-viable or dead cells (or components thereof). If the strain is non-viable the dosage is determined while the strain is still alive/viable. As already mentioned, in one embodiment the compositions may further comprise one or more additional micro-organisms in suitable amounts, such as at least a *Bifidobacterium* (selected from *B. breve*, *B. longum*, *B. lactis*, *B. animalis*, *B. adolescentis*, *B. infantis* and *B. bifidum*), a *L. plantarum* strain and/or a yeast strain (e.g. of the genus *Saccharomyces*). Also mixtures of microorganisms of the invention may be used.

Dosage units may be suitable for various administration regimes. For example, the dosage unit may be administered only once per day or week, or several times per day, such as every 10, 15, 20, 25 or 30 minutes, every hour, every 2, 3, 4, 5, or 6 hours, etc. The dosage unit and administration regime should be such that the desired effect on intestinal MTP-gene modulation is achieved. Routine experimentation can be used to

determine the optimal dosage unit and administration regime (i.e. period of exposure of the intestinal cells to the MTP modulator).

Particular combinations of strains (or their components) may have a synergistic effect with respect to up- or down-regulation of intestinal MTP expression. Therefore, combinations of strains are preferred if these show synergistic effects. Synergy can be defined as an effect of combined strains which is greater than the sum of the effects of the individual strains.

The composition may also be in the form of a freeze-dried powder of the strain or its components (and optionally one or more further microorganisms as described), which can be in the form of a sachet, or which can be incorporated in a capsule or a tablet or another dry administration form. These freeze-dried preparations can be obtained using known techniques and can contain suitable adjuvants known *per se*, for instance cryoprotectants such as maltose. A freeze-dried powder is reconstituted using a suitable liquid, such as water, oral rehydration solution, milk, fruit juice, or similar drinkable liquids. It can also be in the form of a powder which is mixed with solid foods, or foods with a high water-content, such as fermented milk products, for example yoghurt.

The nutritional preparations of the invention can also be in the form of a food which is ready for consumption. Such a food can, for instance, be prepared by adding a strain or a composition comprising a strain of the invention as described above to a food or food base known *per se*, or by adding the microorganism(s) or components thereof (separately or as a mixture) in the amounts required for administration to a food or food base known *per se*; or by cultivating the required bacteria in a food medium until a food containing the amount of bacteria required for administration is obtained. This medium is preferably such that it already forms part of the food, or will form part of the food after fermentation. In this respect, the nutritional composition can be either fermented or non-fermented.

Clearly, the nutritional preparation of the invention can further comprise all desired components, and/or additives which are suited for use in food or food supplements, including flavours, colourings, preservatives, sugar etc. When live or viable microorganisms are used it may be preferred for certain applications that the further

components do not affect the viability of the micro-organisms present therein. In addition other bioactive compounds, such as drugs, medicaments, etc. may be present in the compositions. Also, protective layers may be added around the microorganims, to allow passage into the intestine without affecting viability of the microorganims. For example, acid resistant layers may be added to protect the microorganisms from stomach acids.

As stated, the compositions, especially MTP-inhibitor compositions according to the invention, are suitable for the treatment and/or prevention of weight gain, obesity, atherosclerosis, hyperglyceridaemia, hypercholesterolaemia, diabetes and/or dyslipidaemia, as well as abnormal / sub-optimal intestinal MTP- gene expression levels and protein levels. MTP-activator compositions according to the invention are especially suitable for the treatment and/or prevention of diseases or disorders associated with (too) low intestinal MTP levels, such as abeta- and/or hypobeta-lipoproteinemia or abnormal / sub-optimal intestinal MTP- gene expression levels and protein levels. Also subjects susceptible to developing or suffering from disorders associated with impaired intestinal immune response to antigens or intestinal epithelial cell damage would benefit from administration of an MTP activator composition. In addition, the progression and/or severity of any of the above can be slowed down or reduced using compositions according to the invention.

The administration period depends, of course, on the particular disease or disorder. An administration for several days, weeks, months or even years may be appropriate.

Intestinal MTP expression levels can be monitored at regular intervals in order to determine whether administration should be continued or to modify the administration regime. See also below.

Further aspects of the invention

In addition to the methods described above, it is also an object of the invention to provide a method for establishing an administration (intake) regime and/or a dosage regime for a composition comprising at least one microorganism (preferably a food grade bacterium), said method comprising the steps of:

(a) administering one or more dosages of said composition at one or more time points to a subject or to intestinal tissue or cells of a subject *in vitro* or *in vivo*;

(b) determining the expression levels of the gene encoding Microsomal Triglyceride Transfer Protein (MTP) in said intestinal tissue or cells provided from said human subject at one or more time points after administration or contact, and optionally prior to administration; and

(c) selecting a dosage and/or an administration regime which modulates MTP expression levels in the intestinal tissue or cells in the desired direction (upregulates or down-regulates MTP expression).

This method can be applied not only to the compositions comprising or consisting of one or more microorganisms according to the invention, but also to other food grade microorganisms and compositions comprising these. Without limiting the invention, the underlying theory is that inappropriate consumption (e.g. over-consumption) or uncontrolled intake of certain microorganisms (which either activate or inhibit MTP expression) may lead to an inadvertent activation or downregulation of intestinal MTP expression. Undesired MTP activation may increase the risk of developing obesity, weight gain, obesity, atherosclerosis, hyperglyceridaemia, hypercholesterolaemia, diabetes and/or dyslipidaemia, and/or an increased progression and/or severity of these. In case of MTP over-activation, the dosage regime in step (c) should be such that MTP expression levels are reduced. This may be done by either controlling the intake of the microorganisms which led to the MTP induction in the first place and/or by providing suitable dosages of microorganisms capable of reducing MTP expression, as described herein.

Undesired low MTP levels may increase the risk of disorders associated with impaired intestinal immune response and/or an increased progression or severity of thereof or a higher susceptibility of the intestinal epithelium to damage.

In case of under-activation of MTP expression, the dosage regime/administration regime in step (c) should be such that MTP expression levels are increased. This may be done by either controlling the intake of the microorganisms which led to the MTP inhibition or by providing suitable dosages of microorganisms capable of induce MTP

expression, as described herein. Thus, a regime which counteracts the undesired MTP expression can be established, whereby MTP expression is modulated into the desired direction.

By reducing the dietary intake of strains which have an undesired effect on MTP expression levels, or food compositions comprising these, the risk of developing such diseases, or the progression or severity thereof, can be reduced or prevented.

In the method any composition as earlier defi ned herein (e.g. milk, yoghurt, etc.) comprising one or more microorganisms can be tested. The effect of different (daily) dosages on intestinal MTP expression can then be compared, and for each person an individual, optimal intake regime can be established. Thus, the dosage which leads to MTP up-regulation can be established and the intake can then be kept below this dosage or counteracted by intake of a microorganism having the opposite effect on MTP expression. *Vice versa*, the dosage which leads to MTP down-regulation can be established and the intake can be kept below this dosage or counteracted by intake of a microorganism having the opposite effect on MTP expression.

In a preferred method as defined herein, the expression level of a gene encoding MTP is determined in step b) by analyzing the level of SEQ ID NO: 1, or of a nucleic acid sequence comprising at least 80% sequence identity to SEQ ID NO: 1, in said tissue or cells.

MTP as a biomarker for intestinal health

Also provided is a method for determining the presence or absence of a healthy intestinal microbiota in a human subject, wherein intestinal MTP gene expression levels are used as a biomarker for the presence or absence in the human intestine of probiotic bacteria in beneficial amounts. For using MTP as a biomarker, the MTP gene expression levels are preferably analysed *ex vivo* using a biological sample (intestinal tissue or cells) of a human subject. Alternatively *in vivo* methods for assessing MTP expression levels may be used.

The method comprises the assessment of MTP expression levels and/or protein levels, as described.

Thus, an *in vitro* method for monitoring or assessing the health of the intestinal microbiota of a human subject is provided, comprising:

a) measuring MTP-gene expression levels and/or protein levels in a first biological sample from a healthy subject;
b) measuring MTP-gene expression levels and/or protein levels in a second biological sample from a patient;
c) determining the difference in MTP-gene expression levels and/or protein levels between the first and second biological sample, wherein a significant difference is indicative of a deviant intestinal microbiota and sub-optimal or abnormal intestinal health.

The test subjects (patients) may be healthy subjects or subjects suffering from one or more symptoms of gastrointestinal illness or disorders. The MTP expression levels of patients are compared to those found in healthy human subjects in order to determine whether they deviate in any way therefrom (e.g. whether they are higher or lower). Too high or too low levels (relative to the normal expression levels) is indicative of a sub-optimal intestinal health and sub-optimal microbiota.

The biological sample is preferably a cell or tissue sample of the intestine. It is understood that a) and b) preferably involves the analysis of several subjects and/or several biological samples, for example taken at different time points.

Thus, too high MTP expression levels may be indicative of insufficient probiotic bacteria capable of downregulating MTP expression and/or of a risk of developing one or more disorders selected from the group of weight gain, obesity, atherosclerosis, hyperglyceridaemia, hypercholesterolaemia, diabetes and/or dyslipidaemia. Too low MTP expression levels may indicative of insufficient probiotic bacteria capable of upregulating MTP expression and/or of a risk of developing disorders associated with impaired intestinal immune response to antigens and/or of a higher susceptibility of the intestinal epithelium to damage.

In a preferred method, the expression level of an MTP gene and/or protein is measured in step b) by analyzing the level of SEQ ID NO: 1, or of a nucleic acid sequence comprising at least 80% sequence identity to SEQ ID NO: 1, in said tissue or cells.

In a further embodiment kits are provided for monitoring or assessing the health status of the intestinal microbiota of human subjects. Such kits comprise for example primers or probes for determining MTP gene expression levels in human intestinal cells/tissues (such as fragments of SEQ ID NO: 1 or 3, or variants thereof; e.g. SEQ ID NO: 4-14), antibodies for detecting MTP protein levels, nucleic acid carriers (for example be arrays and microarrays or DNA chips, comprising nucleotides on a glass, plastics, nitrocellulose or nylon sheets, silicon or any other solid surface, which are well known in the art), buffers, regents, etc. and preferably biological (intestinal cell/tissue) samples or nucleic acid and/or protein from such biological samples, which are suitable as reference samples for determining the amount of MTP mRNA or protein in a test sample.

Sequences

SEQ ID NO 1: mRNA of the human MTP gene (X91148)

SEQ ID NO 2: amino acid sequence of the human MTP protein (CAA62585.1)

SEQ ID NO 3: target MTP mRNA fragment of Affymetrix gene chip U133A.

SEQ ID NO 4-14: probes which hybridize with SEQ ID NO: 3.

Unless stated otherwise, the practice of the invention will employ standard conventional methods of molecular biology, virology, microbiology or biochemistry. Such techniques are described in Sambrook *et al.* (1989) Molecular Cloning, A Laboratory Manual (2nd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press; in Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual,* Third Edition, Cold Spring Harbor Laboratory Press, NY; in Volumes 1 and 2 of Ausubel *et al*. (1994) *Current Protocols in Molecular Biology, Current Protocols*, USA; and in Volumes I and II of Brown (1998) *Molecular Biology LabFax*, Second Edition, Academic Press (UK); *Oligonucleotide Synthesis* (N. Gait editor); *Nucleic Acid Hybridization* (Hames and Higgins, eds.), all incorporated herein by reference.

Examples

1. Material and Methods

1.1 Preparation *of Lacobacillus plantarum* WCFS1

Lactobacillus plantarum WCFS1 was grown on MRS medium under anaerobic conditions. Fifteen minutes prior to each experiment with *L.plantarum* WCFS1, 10^{11} freshly prepared *Lactobacillus plantarum* WCFS1 were resuspended in 600 mL saline and 10 g/L glucose, and kept on 37°C in a heated, gently shaking water bath until administration to the subject.

Effects of *Lactobacillus plantarum* WCFS1 on small intestinal mucosa were investigated in two human intervention studies, study 1 (short term exposure) and study 2 (long term exposure).

1.2 Study 1 – short term exposure (1 hour)

In the first study, eight healthy volunteers (24 ± 4y) without a history of GI symptoms were tested on two separate occasions in a randomised cross-over study. After an overnight fast, duodenal biopsies from the horizontal part of the duodenum, at approximately 15 cm distal to the pylorus, were obtained by standard flexible gastroduodenoscopy. After obtaining the mucosal samples, a guide wire was inserted with the distal tip positioned about 60 cm distal to the pylorus, facilitated by gastroduodenoscopy. Subsequently, the endoscope was retracted leaving the guide wire in the gastrointestinal tract, and a double lumen perfusion catheter (home made from PVC tubing, inner diameter 2.5 mm, outer diameter 4 mm) was inserted over the guide wire into the proximal small intestine with the tip of one lumen, which served as an injection port, located in the proximal descending duodenum and the tip of the second lumen, with three small holes located in the distal 3 cm of the catheter to serve as a sample port, 40 cm distal to the injection port. After positioning, the guide wire was removed. The procedure was performed under short interval fluoroscopic control. The 40-cm segment of the proximal small intestine was perfused with saline and 10 g/L glucose for 180 min at 10 ml/min using a peristaltic pump to obtain steady state conditions. After reaching steady state, saline with 10 g/L glucose with or without, in total, $1x10^{11}$ *Lactobacillus plantarum* WCFS1 was infused for one hour. Food or beverage consumption was not allowed during the experiment. Fifteen minutes after cessation of the perfusion experiment, the perfusion catheter was removed and a second

gastroduodenoscopy was performed to obtain tissue samples from the same intestinal region after the intestinal perfusion experiment. These tissue samples were obtained 3-4 cm distally from those taken in the morning. The exact position of the tissue sampling in the morning was achieved by visual inspection of the gut mucosa. A red colour from the mucosa, different from the surrounding tissue, showed the exact position of earlier tissue sampling. The entire protocol was repeated on another day, one to two weeks after the first experiment, to randomly investigate the effects of placebo or *Lactobacillus plantarum* WCFS1.

In all tissue samples, gene expression levels were measured using genome-wide microarrays (Affymetrix U133A) as described below.

1.3 Study 2 – long term exposure (6 hours)

In the second study, seven healthy volunteers were recruited to participate in another randomized crossover study. After an overnight fast, an intraduodenal feeding catheter (naso-intestinal tube, Flocare® Bengmark, Nutricia Healthcare S.A., Chatel-St.Denis, Switzerland) was placed orogastrically conform the manufacturers' instructions manual. Briefly, the feeding tube was inserted nasogastrically. It emptied from the stomach into the small intestine by normal peristaltic movements. After positioning of the catheter in the small intestine (tube tip positioned 5-10 cm distal to the pylorus), a test solution containing in total 10^{12} *Lactobacillus plantarum* WCFS1 and 10 g/L glucose in saline or, randomly on another test day, only 10 g/L glucose in saline, was injected continuously at 6.7 ml/min for 6 h. Subjects remained in the supine position until the end of the experiment. Food or beverage consumption was not allowed during the experiment. After this 6-h period, a tissue sample was obtained at approximately 15 cm distal to the pylorus by standard flexible gastroduodenoscopy. In all tissue samples, gene expression levels were measured using genome-wide microarrays (Affymetrix U133A) as described below. Additionally, in duplicate tissue samples, differential proteome analyses were performed using the CyDIGE method (2D gel-electrophoresis with minimal fluorescent labelling) with Maldi-TOF identification as described below.

1.4 Study 3 – long term exposure (6 hours) after oral intake

In the third study, eight healthy volunteers were recruited to participate in a randomized double-blind cross-over study with four interventions. After an overnight fast, volunteers were instructed to drink beverages containing a test drink at 30-min intervals during 6 consecutive hours. On the day of testing, subjects were not allowed to consume any food or other beverages. The beverages consisted of one of the following test drinks:

- 1% glucose / 8% maltodextrin (placebo)
- 10^{12} stationary phase of growth-harvested *Lactobacillus plantarum* WCFS1 (in 1% glucose / 8% maltodextrin)
- 10^{12} non-viable, stationary phase of growth-harvested *Lactobacillus plantarum* WCFS1, killed by heating to 80°C (in 1% glucose / 8% maltodextrin)
- 10^{12} mid-logarithmic phase of growth-harvested *Lactobacillus plantarum* WCFS1 (in 1% glucose / 8% maltodextrin)

After the 6-h period, tissue samples from the proximal small intestine were obtained by standard flexible gastroduodenoscopy. In the samples, gene expression profiles were determined with genome-wide Affymetrix HG U133 Plus 2.0 microarrays.

1.4 Microarray analysis

Ribonucleic acid (RNA) isolation was performed using commercial kits (RNeasy Kit; Qiagen, Hilden, Germany), following the manufacturers protocol. Gene expression was examined using the GeneChip technology (Affymetrix). Briefly, biotin-labelled cRNA was generated by in vitro transcription and hybridized to the GeneChips (HG-U133A for studies 1 and 2; HG-U133 Plus 2.0 for study 3) following the manufacturers instructions. Fragmentation of cRNA, hybridization to GeneChips, washing and staining, as well as scanning of the arrays in the GeneArray scanner (Agilent) were performed as recommended by the Affymetrix Gene Expression Analysis Technical Manual.

Images of the Human Genome U133A and Human Genome U133 Plus 2.0 arrays were quantified with GCOS software (Affymetrix) respectively for the Perfusion experiments and the Oral intake experiment. Further in the Oral intake experiment, the chip description file (CDF) used for the analysis was an update created and freely

distributed by the microarray lab of the university of Michigan based on UniGenes (version 7). A more detailed description of this analysis is shown below in the supplementary data. Briefly for Perfusion 1, the genes were analyzed using a multivariate Gaussian linear regression including the hybridization and labeling spikes, the test day, and the perfusion procedure. For Perfusion 2, the genes were analyzed using a multivariate Gaussian linear regression including the hybridization and labeling spikes. For Oral intake, the genes were analyzed using a multivariate Gaussian linear regression including the hybridization and labeling spikes, and the hybridization day. The inference criterion used for comparing the models is their ability to predict the observed data, i.e. models are compared directly through their minimized minus log-likelihood. When the numbers of parameters in models differ, they are penalized by adding the number of estimated parameters, a form of the Akaike information criterion (AIC) (Akaike H (1973) Information theory and an extension of the maximum likelihood principle. In: Petrov BN, Csàki F (eds) Second International Symposium on Inference Theory. Akadémiai Kiadó, Budapest, p 267-281). For each gene of Perfusion 1, the treatment was then accounted for in the model by adding the interaction between the test day and perfusion procedure. For each gene of Perfusion 2, the treatment group was then added to the model. For each gene of the Oral intake, the groups (placebo, stationary *Lacobacillus plantarum*, mid-log or diluted *Lacobacillus plantarum*, heat killed) were then added to the model. The gene under consideration was found to be differentially expressed if the AIC decreased compared to the model not containing the treatment or group effect.

The genes analyzed and fold changes were loaded into GenMapp (Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC, Conklin BR (2002) GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. Nat Genet 31:19-20) and MAPPFinder (Doniger SW, Salomonis N, Dahlquist KD, Vranizan K, Lawlor SC, Conklin BR (2003) MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. Genome Biol 4:R7) software packages to evaluate the transcripts in relation to known biological processes, molecular function and cellular component based on Gene Ontology (GO) terms (Ashburner et al., 2000) and local maps. Only gene-transcripts with either their average intensities for the placebo and treated groups above 500 or average intensities for one

of these groups above 1000 and a 10 percent up or down regulation fold change were used to obtain a ranked list of pathways with differentially expressed genes.

MappFinder software was used to select the MAPPs with relatively high numbers of differentially expressed genes, which were affected by *Lacobacillus plantarum* WCFS1 compared to placebo. The ranking of regulated pathways was indicated by the individual Z-scores. The Z-score increases when higher numbers of changing genes are found, taking into account the number of genes present in the MAPP that are represented on the array, and the total number of genes involved in the concerning MAPP. MAPPs were selected for further study if the group results (*Lacobacillus plantarum* WCFS1 compared to placebo) reached an arbitrary Z-score of at least 2 on that MAPP, and at least 3 genes were differentially expressed in that pathway.

Supplementary data Microarray analysis

Only in the case of the Oral intake experiment, the chip description file (CDF) used for the analysis was an update created and freely distributed by the microarray lab of the university of Michigan [10, 11] based on UniGenes (version 7). This resulted in the analysis of 19278 gene-transcripts out of the 54613 commonly obtained using the Human Genome U133 Plus 2.0 CDF provided by Affymetrix.

All gene-transcripts were analysed using a multivariate Gaussian linear regression ($MVN(\mu, \Sigma)$) where μ is the mean, Σ is the covariance matrix $\begin{pmatrix} \sigma^2+\delta & \delta & \cdots & \delta \\ \delta & \ddots & \ddots & \vdots \\ \vdots & \ddots & \ddots & \delta \\ \delta & \cdots & \delta & \sigma^2+\delta \end{pmatrix}$, σ^2 is the variance, and δ as both the extra component of variance across subjects and the common covariance among responses on the same subject) including the hybridization and labeling spikes, and a random effect for Perfusion 2, additionally the test day (placebo or *Lacobacillus plantarum*) and the perfusion procedure (before or after the procedure) for Perfusion 1, or for the Oral intake the best hybridization and best labeling spike, the hybridization day, and a random effect. The inference criterion used for comparing the models is their ability to predict the observed data, i.e. models are compared directly through their minimized

minus log-likelihood. When the numbers of parameters in models differ, they are penalized by adding the number of estimated parameters, a form of the Akaike information criterion (AIC) [1].

For each gene of Perfusion 1, a model containing the relevant covariates mentioned above ($E(y) = Spike_1 + \ldots Spike_n + Test + Perfusion$) was fitted in order to obtain a reference AIC. Then a model containing the treatment effect was fitted by adding the interaction between the test day and the perfusion procedure (Perfusion) ($E(y) = Spike_1 + \ldots Spike_n + Test + Perfusion + Test \times Perfusion$). For each gene of Perfusion 2, a model containing the relevant covariates mentioned above ($E(y) = Spike_1 + \ldots Spike_n$) was fitted in order to obtain a reference AIC. Then a model containing the treatment group (Grp) was fitted ($E(y) = Spike_1 + \ldots Spike_n + Grp$). For each gene of Oral intake, a model containing the relevant covariates mentioned above ($E(y) = Hyb + Spike.Lab + Spike.Hyb$) was fitted in order to obtain a reference AIC. Then a model containing the groups (placebo, stationary: S, mid-log: ML, and dead: D) was fitted ($E(y) = Hyb + Spike.Lab + Spike.Hyb + Grp + S.Grp + ML.Grp + D$). The gene under consideration was found to be differentially expressed if the AIC of this second model decreased compared to the model not containing the treatment or groups.

1.5 Proteomics analysis

Intestinal biopsies were prepared for protein analysis by sonification in a lysis buffer, and subjected to the 2-D Clean Up kit (Amersham Biosciences, Freiburg, Germany) to remove non-protein material. Protein concentrations were measured using the 2-D Quant Kit (Amersham Biosciences, Freiburg, Germany). The samples were further processed for two-dimensional fluorescence difference gel electrophoresis with the Ettan™ DIGE technique, following the manufacturers' instructions [30]. Briefly, samples were stained with both CyDye DIGE Fluor Cy5 minimal dye and CyDye DIGE Fluor Cy3 minimal dye, and a pooled internal standard, containing equal amounts of all protein samples from this experiment, with CyDye DIGE Fluor Cy2 minimal dye, according to the kits instructions. Protein mixtures were separated by 2D electrophoresis, according to their iso-electric points, using the Ettan IPGphor 3

isoelectric Focusing System, and to their molecular weights using an EttanDalt12 electrophoresis system. Samples from the placebo intervention and from the *L. plantarum* intervention were loaded on one IPG-strip, together with the pooled internal standard. After the 2D-electrophoresis, the gels were scanned with a Typhoon confocal laser scanner (Typhoon 9410 Variable Mode Imager, Amersham Biosciences), and the scanned images were loaded into DeCyder software (Decyder 2D, Amersham Biosciences, Freiburg, Germany) to analyse the differences in protein profiles. Differentially expressed protein spots were picked with the Ettan Spot picker (Amersham Biosciences, Freiburg, Germany). Excised spots were subjected to mass-fingerprint identification using Maldi-TOF MS analysis.

1.6.1 Q-PCR

First Strand cDNA was synthesized using the ReactionReady™ First Strand cDNA Synthesis Kit (Superarray, USA) according to the manufacturer's instructions. Briefly, 500 ng total RNA was used as template for the reaction. To synthesize the cDNA, Random Primers, RNase inhibitor and Reverse Transcriptase were used according to the manufacturer's instructions. Subsequently, the cDNA was diluted with RNase free H_2O to 5 ng/µl. Each well of the 96 well PCR array plate (Superarray, USA) contained 1 µl gene-specific 10 µM PCR primer pair stock. To each well 24 µl PCR array reaction mixture was added. This PCR Array reaction mixture contained 98 µl diluted template cDNA, 525 µl RT^2 Real-Time™ Sybr Green/ROX PCR Master Mix and 527 µl RNase free H_2O (Superarray, USA). Reactions were run on an ABI PRISM 7000 Sequence detection System (Applied biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The cycling conditions comprised 10 minutes 95°C and 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. On each plate two samples were run, each for 36 different genes of interest, 10 different housekeeping genes, a total RNA control and a no template control. Data was analysed with the 7000 System Sequence detection Software, version 1.2.3 (Applied Biosystems).

1.6.2 Q-PCR calculations

Study 1. Gene-transcripts were analyzed using a multivariate Gaussian linear regression, similar to the microarray analysis, with the difference of having the sample concentration, the test day, the perfusion procedure, repeats, 18S ribosomal RNA, and the best housekeeping gene among ACTB, ACTB-RTC, CANX, DDX5, GAPD, ILF2,

NARS, PRPF8, SART1, and TMED2 included. For each gene, the treatment was then accounted for in the model by adding the interaction between the test day and perfusion procedure. The AIC was also used to asses whether there was a treatment effect. Hence, the gene under consideration was found to be differentially expressed if the AIC decreased compared to the model not containing the interaction.

Study 2. Gene-transcripts were analyzed using a multivariate Gaussian linear regression, similar to the microarray analysis, with the difference of having repeats, 18S ribosomal RNA and the best housekeeping gene among ACTB, ACTB-RTC, CANX, DDX5, GAPD, ILF2, NARS, PRPF8, SART1, and TMED2 included. For each gene, the treatment group was then added to the model. The AIC was also used to asses whether there was a treatment effect. Hence, the gene under consideration was found to be differentially expressed if the AIC decreased compared to the model not containing the treatment difference.

Example 2 - Results

2.1 Study 1 – short term exposure

We observed significant changes in 689 gene reporters; 225 genes were upregulated, and 444 gene reporters were downregulated. Most of the observed changes were small with regard to the robust change, but changes were consistent for all volunteers.

The pathway analysis software GenMapp was used to identify regulated biological pathways. We identified 40 pathways that had a Z-score > 2, and in which 3 genes or more were differentially expressed. The gene reporter that represented MTP, with Affymetrix reference number 205675_at and UniGene reference Hs.195799, was down-regulated by 14.8 % by the short-term *Lactobacillus plantarum* WCFS1 intervention study, Table 1). This downregulation indicates a potentially important biological effect of the bacterial intervention. The downregulation was confirmed by Q-PCR in 4 out of 8 subjects.

2.2 Study 2 – long term exposure

The 6-h *Lactobacillus plantarum* WCFS1 challenge significantly mediated the expression of 424 gene reporters; 383 were upregulated and 41 were downregulated.

The pathway analysis software GenMapp was used to identify regulated biological pathways. These ranked lists showed that, based on Gene Ontology databases, 52 pathways had a Z-score > 2, and 3 genes or more were differentially expressed.

The proteome analyses showed that two proteins differed consistently in all volunteers. One of these spots was identified as microsomal triglyceride transfer protein (MTP), while this analysis failed to allow identification of the second spot. The gene expression analysis showed that the gene encoding this protein, which was identified on the Affymetrix U113A microarray with the identification reference number 205675_at and UniGene reference Hs.195799, was significantly upregulated by 19.3 %, as a result of the *Lactobacillus plantarum* WCFS1 exposure (Table 1). The upregulation was confirmed by Q-PCR in 5 out of 7 subjects.

2.3 Study 3 – long term exposure after oral intake

The 6-h oral intake of *Lactobacillus plantarum* WCFS1, non-viable *Lactobacillus plantarum* WCFS1, and mid-log harvested *Lactobacillus plantarum* WCFS1 resulted in mediation of several hundreds of genes. The gene reporter representing the MTP gene is not differentially expressed by any of the microbe interventions when compared to placebo. However, the stationary or fully grown *Lactobacillus plantarum* WCFS1 induced an increase of 13.9 % (P = 0.179) in MTP expression (Table 1). The magnitude of upregulation is comparable to that observed after 6-h intraluminal exposure to *Lactobacillus plantarum* WCFS1 (study 2). Mid-log harvested *Lactobacillus plantarum* WCFS1 and non-viable *Lactobacillus plantarum* WCFS1 did not modulate MTP expression.

Table 1. Changes in MTP expression after exposure to *Lactobacillus plantarum* WCFS1, compared to placebo

Study 1 Intraluminal administration for 1 h	Study 2 Intraluminal administration for 6 h	Study 3 Oral administration for 6 h
14.6 % downregulation	19.3 % upregulation	13.9 % upregulation

2.3 Conclusion

These three human intervention studies showed that administering *Lactobacillus plantarum* WCFS1 to the small intestine in healthy volunteers *in vivo* results in changes in expression of the gene 'microsomal triglyceride transfer protein' (MTP). This was accompanied by a significant change in the microsomal triglyceride transfer protein.

CLAIMS

1. Use of a food-grade bacterium capable of modulating Microsomal Triglyceride Transfer Protein (MTP) gene expression in human intestinal tissue or intestinal cells for the preparation of a composition for the treatment or prevention of weight gain, obesity, atherosclerosis, hyperglyceridaemia, hypercholesterolaemia, diabetes, dyslipidaemia and/or disorders associated with impaired intestinal immune response to antigens.

2. A use according to claim 1, wherein said food-grade bacterium is a probiotic bacterium and/or a lactic acid bacterium, preferably selected from one of the following genera: *Lactobacillus, Streptococcus, Lactococcus, Oenococcus, Leuconostoc, Pediococcus, Carnobacterium, Propionibacterium, Enterococcus* and *Bifidobacterium*.

3. A use according to claim 1 or 2, wherein said food-grade bacterium is capable of upregulating or downregulating said MTP gene expression.

4. A use according to claim 3, wherein said MTP gene expression is upregulated or downregulated by at least 10% following contact with said bacterium.

5. A use according to any one of the preceding claims, wherein said bacterium is either a live or viable bacterium.

6. A use according to any one of the preceding claims wherein the modulation of MTP gene expression is determined by analyzing the level of SEQ ID NO: 1, or of a nucleic acid sequence comprising at least 80% sequence identity to SEQ ID NO: 1, in said tissue or cells.

7. A method for identifying or selecting a food grade bacterium capable of modulating intestinal MTP gene expression, comprising:

(a) contacting a composition comprising or consisting of a food grade bacterium with intestinal tissue or intestinal cells of a human subject *in*

vitro or using a biological sample of intestinal tissue or intestinal cells that had been contacted with said composition *in vivo*;

(b) determining the expression level of the gene encoding human Microsomal Triglyceride Transfer Protein (MTP) in said tissue or cells; and

(c) identifying or selecting a food grade bacterium which upregulates or downregulates MTP gene expression levels in said tissue or cells.

8. A method according to claim 7, wherein the modulation of an intestinal MTP gene expression is determined in step b) by analyzing the level of SEQ ID NO: 1, or of a nucleic acid sequence comprising at least 80% sequence identity to SEQ ID NO: 1, in said tissue or cells.

9. A method according to claim 7 or 8, wherein said food grade bacterium is a probiotic and/or lactic acid bacterium.

10. A food, food supplement or feed composition comprising or consisting of a suitable amount of a food grade bacterium, characterized in that said bacterium is capable of modulating intestinal MTP gene expression levels in a human subject *in vivo*.

11. A food or food supplement composition according to claim 10, wherein said composition is a liquid, a solid or a semi-solid.

12. A food or food supplement composition according to claim 10 or 11, wherein said composition is a dairy product, preferably a yoghurt, cheese or milk-based drink.

13. A method for establishing an administration regime and/or a dosage regime for a composition comprising or consisting of at least one food grade bacterium, said method comprising the steps of:

(a) contacting one or more dosages of said composition at one or more time points with intestinal tissue or intestinal cells of a human subject *in vitro* or *in vivo*;

(b) determining the expression levels of the gene encoding Microsomal Triglyceride Transfer Protein (MTP) in said intestinal tissue or cells provided from said human subject at one or more time points after contact; and

(c) selecting a dosage and/or an administration regime which modulates MTP gene expression levels in said intestinal tissue or cells.

14. A method according to claim 13, wherein the expression level of a gene encoding MTP is determined in step b) by analyzing the level of SEQ ID NO: 1, or of a nucleic acid sequence comprising at least 80% sequence identity to SEQ ID NO: 1, in said tissue or cells.

15. A method according to claim 13 or 14, wherein said administration regime and/or dosage regime is for a composition for the treatment or prevention of weight gain, obesity, atherosclerosis, hyperglyceridemia, hypercholesterolaemia, diabetis, dyslipidaemia, disorders associated with impaired intestinal immune response to antigens and/or a sub-optimal or abnormal intestinal microbiota.

16. A method according to any one of claims 13 to 15, wherein the composition is a food, food supplement or feed composition.

17. An *in vitro* method for monitoring or assessing the health status of the intestinal microbiota of a human subject is provided, comprising:

a) measuring MTP-gene expression levels and/or protein levels in a first intestinal tissue sample from a healthy subject;

b) measuring MTP-gene expression levels and/or protein levels in a second intestinal tissue sample from a patient; and

c) determining the difference in MTP-gene expression levels and/or protein levels between the first and second tissue sample, wherein a significant difference is indicative of a deviant intestinal microbiota and sub-optimal or abnormal intestinal health.

18. An *in vitro* method according to claim 17, wherein the expression level of an MTP gene and/or protein is measured by analyzing the level of SEQ ID NO: 1, or of a nucleic acid sequence comprising at least 80% sequence identity to SEQ ID NO: 1, in said tissue.

19. A method according to claim 17 or 18, wherein a significantly higher MTP gene expression level and/or protein level in the patient relative to the healthy subject is indicative of insufficient intestinal probiotic bacteria capable of downregulating MTP expression and of a risk of developing one or more disorders selected from the group of weight gain, obesity, atherosclerosis, hyperglyceridemia, hypercholesterolaemia, diabetes and/or dyslipidaemia and wherein a significantly lower MTP gene expression level and/or protein level in the patient relative to the healthy subject is indicative of insufficient probiotic bacteria capable of upregulating MTP expression and of a risk of developing disorders associated with impaired intestinal immune response to antigens and a high susceptibility of the intestinal epithelium to damage.

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2007/050667

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61P1/00 A61P3/04 A61P9/00 A61P9/10 A61P3/08
A61P3/10 A61P1/14 A61P37/00 A61K35/74

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/025643 A (PL BIO CO LTD [KR]; CJ CORP [KR]; LEE YEON-HEE [KR]; PAEK KYUNG-SOO [K) 9 March 2006 (2006-03-09) claims 10-13	1-5,10
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 January 2008	07/02/2008

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2007/050667

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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